Sept 27, 2005

Health Care Without Harm comments on:

DRAFT:
NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl)phthalate
Aug, 2005

Health Care Without Harm (HCWH) submits the following comments on the August 15, 2005 draft document "NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl)phthalate" and encourages the panel to consider them in their final evaluation of the reproductive and developmental toxicity of DEHP.

Summary of HCWH's major comments:

The draft report update does not indicate the criteria used for deciding whether or not to include data in the evaluation process. Specifically, the report describes data presented in several unpublished studies but does not indicate if or the extent to which the panel reviewed the original data from those studies. This is particularly important since unpublished studies have not undergone peer review. If the panel considers unpublished studies in the final evaluation of DEHP, panel members should assure themselves that study design, data gathering, statistical analysis, and interpretation are supportable and conform to recognized standards.

In particular, HCWH strongly encourages the panel to review the raw data, statistical analysis, and interpretation of the 65-week marmoset feeding study (Mitsubishi) and the 2-generation rodent reproductive toxicity study (Schilling). More detailed comments below describe the rationale for more thorough scrutiny of those data, which includes: 1) the Mitsubishi authors' failure to consider some data, for reasons that are unconvincing and not supported by the data; 2) their failure to assess a significant amount of tissue histologically, as called for in the study protocol; and 3) failure of the draft document to comment on histological data in Schilling et al.

A discussion of the limitations of the Mitsubishi and Schilling studies should be added to the "Strengths/Weaknesses" and "Utility (Adequacy) for CERHR Evaluation Process" summaries—pg. 113 for Mitsubishi and pg. 114 for Schilling.

Comments by section and page number:

Sect. 1.0 Human Exposure

Page 4—"Abstracts noted for completeness but are not used in the evaluation process"

This comment shows up in several places in the draft report. What are the criteria for using information in the evaluation process?

Page 20—The draft report says that "not all investigators agree with the methods used to derive these estimates, and alternative estimates have been as much as 5-fold lower" (ref 25) It would be more accurate to say that one investigator does not agree with these estimates. That alternative estimate is discussed in the published exchange between Koch and David referenced earlier in the draft

Sect 2.0—General toxicology and biologic effects

Pg 25-26—table 14, 15. These tables summarize toxicokinetic data from the 65-week marmoset feeding study (Mitsubishi).

A review of raw data from the Mitsubishi study shows several important factors worth noting:

The data from this study in marmosets show considerable variability in the timing, extent of uptake, and excretion patterns over the 168 hour study period. For example, there is significant individual variability with respect to Cmax and Tmax in both dose groups, particularly in the 3 month old marmosets. In Table 15, the organ/plasma ratio is based on the mean testicular level/mean plasma level at 2 hrs post dosing. However, the raw data show that one of three of the 3 month old animals had a Tmax of 8 hrs, while the other two had a Tmax of 2 hrs. Calculations of organ/plasma ratios at a fixed time will miss the individual variability in toxicokinetics.

The raw data also indicate substantial individual variability in reproductive organ radioactivity content. The standard deviation is larger than the mean for testis, epididymis, and uterus in the 3 month old animals.

More importantly, these observations are consistent with previously recognized individual variability in outcomes in rodent toxicity studies and suggest that susceptibility to developmental impacts may be significantly influenced by variable absorption of DEHP and/or MEHP from the intestinal tract as well as variable tissue sensitivity.

Variability in blood levels was also noted in the study by Kessler et al. comparing AUCs in marmosets and rats. Cmax in rats was on average 3.2 times higher than marmosets but the range was 1.3-7.5. The AUC in rats was on average 7.3 higher than marmosets but the range was 2.6-15.6. It should also be noted that each time point in the sampling curve for marmosets in Kessler et al. is based on data from a single animal. Consequently, these data do not provide information about individual variability among marmosets—a matter of significant importance when interpreting the Mitsubishi marmoset study as discussed below.

Kessler et al. also note that the AUC reported in their study was one order of magnitude lower than that calculated from the AUC of DEHP published by Pollack et al. (1985).

They suggest that the difference might result from the difference in vehicles used, a lipophilic one (corn oil) by Pollack and hydrophilic one by Kessler et al..

Page 8 of the draft report summarizes findings from Koch et al. which reported urine and blood measurements of DEHP metabolites in a 61-yr old German male who ingested 3 different doses of labeled DEHP. As noted, the maximum concentration of MEHP in his blood was of the same order of magnitude as in animal studies, although the dose was 50-1000 times lower than in the animal studies. Koch et al. also noted, however, that the dose-normalized AUC was about 15-100 times higher in the human volunteer than in the rats and marmosets, respectively, using the same method for estimating the AUC. This suggests that humans absorb DEHP/MEHP much more readily than marmosets.

It is generally accepted that DEHP administered orally is metabolized into MEHP by non-specific lipases and/or esterases prior to intestinal absorption. Little attention has been paid to variability in that process. Variability would be expected not only because of species differences but also from individual differences, enzyme induction related to timing and type of food intake, and the matrix in which DEHP is ingested. This becomes particularly important when considering the Mitsubishi authors' choices of which animals to include in evaluation of biological endpoints (see below).

Sect 3.0—Developmental toxicity

Page 36—it should be noted in the Swan study that a regression coefficient was calculated for each DEHP metabolite separately. No attempt was made to integrate the metabolites into a single expression of exposure to the parent compound in order to determine whether or not a significant correlation existed between exposure and anogenital index.

Page 36-37—(Rais-Bahrami) It should be added that wide variability in "normal" values for the hormonal and anatomic parameters measured at this age further restrict the value of any conclusions that may be drawn from this study.

Sect. 4.0—Reproductive toxicity

Pg 112—Mitsubishi 65-week feeding study, marmosets:

The 65-week marmoset study (Mitsubishi) began dosing at 90-115 days of age and continued until day 455. Dosing began after weaning and did not include DEHP exposure during the neonatal period and therefore sheds no light on vulnerability during the prenatal period or infancy in marmosets.

It should also be noted that marmosets have adult numbers of Sertoli cells by 18-22 weeks of age (126-154 days) and presumably an intact blood-testis barrier by that time.

Vitamin C supplements were added to the diet during the treatment period. Marmosets require sufficient dietary Vitamin C and are sensitive to Vitamin C deficiency. It is,

therefore, not unusual to provide supplements to captive animals if there is reason to believe that the basic diet may contain insufficient amounts. However, in the Mitsubishi study, the extent to which the basic diet contained Vitamin C in addition to the supplement is not reported. In rodents, Vitamin C is reported to mitigate the testicular toxicity of DEHP, and this should be considered in data interpretation.

Several observations from the raw data collected during this study inevitably lead to the conclusion that the panel must completely evaluate the entire data set from the Mitsubishi study before deciding the extent to which to include the results in the final evaluation.

One animal in each treatment group had dramatic reductions in testes weight at 66 weeks:

Animal:	Dose grp:	#/grp	Testicular weight:
Animal 10204	(100 mg/kg)	6	0.19 gm,
Animal 10302	(500 m/kg)	7	0.22 gm,
Animal 10402	(2500 mg/kg)	6	0.14 gm.

compared to means of:

0.83 gm, (SD 0.14)	(control);
0.73 gm, (SD 0.29)	(100 mg/kg);
0.73 gm (SD 0.27)	(500 mg/kg);
0.75 gm (SD 0.41)	(2500 mg/kg).

These same animals had markedly reduced sperm counts.

The authors noted that "one animal in each treatment group had low weights of testes, epididymis, seminal vesicle, and prostate." They then say "these animals usually showed low body weight and such animal as showing low body weigh [sic] and small size in above organs also existed in the control animals of Group 2."

[From the raw data, one can see that animals 10204, 10302, and 10402 had body weights of 223 gm, 206 gm, 212 gm at the end of the treatment period with group mean body weights of 263 gm, SD 19 (control), 273 gm, SD 54.5 (100 mg/kg), 267.8 gm, SD 44.7 (500 mg/kg), 267 gm, SD 30 (2500 mg/kg).

Animal 10204 did not have the lowest body weight in its group, yet its sperm count data were discarded. Animal 10302 stopped gaining weight about half way through the treatment period. There were no reports of unusual clinical signs in this animal. Animal 10402 was the smallest animal in its treatment group at the outset and remained small throughout the treatment period.

Each control animal gained weight during the treatment period. The smallest control animal weighed 95 gms at the outset and 241 gms at the end of the treatment period. No

control animal had unusually small absolute or relative testicular weight. The authors' assertions to the contrary are not supported by the data.]

The authors also use the low body weight of these animals from the three treatment groups as a rationale for omitting them from Table 7, which reports the sperm count summary data. The authors suggest that since the "occurrence of the growing animal is not dose-dependent,…..the inclusion of exceptional incidence was avoided." That rationale for discarding data is unconvincing. It ignores alternative plausible explanations: 1) these animals are simply at the low end of body weight and/or 2) the observed effects are treatment related and that absorption of the test compound from the GI tract is saturated at the doses used in this study.

Testosterone levels at the end of treatment period show that there is large variability among individuals within each treatment group. The mean values are often driven by one animal in each group whose testosterone level is an order of magnitude different from the others in the same group. This variability is reflected in the large SD in each group.

Of note, animal 10204 had testosterone levels at week 65 that were about half that of the next lowest level in the same treatment group and up to 2 orders of magnitude lower than the highest level in that group.

Animals 10302 and 10402 had pre-treatment testosterone levels that were the highest in their respective treatment groups, but throughout the treatment period their testosterone level dropped beneath and remained beneath the quantitation limit. It is therefore unclear what the authors mean when they say, "during the treatment period, all males experienced a surge in testosterone."

These same three animals (10204, 10302, 10402) were also omitted from any analysis of testicular enzyme activity.

Among those animals that were included in the groups subject to testicular enzyme analysis and histological examination, for completely unexplained reasons, one-third of all male animals (three from each treatment group and controls) did not have the testis histologically examined. All other tissues were examined in all groups, except for the prostate, which was reported as lost in two animals. (one each in the 100 mg/kg and 500 mg/kg groups.)

In one animal in each of the treatment groups, testicular findings were reported as "yes," but attributed to "growing" animal and not further described or considered treatment related. These were animals 10204 (discussed above), 10302, and 10402 (discussed above). As with the other animals, 10302 had essentially stopped gaining weight about half way through the treatment period.

In summary, this study begins with limited power to detect significant effects because of small numbers of animals in each treatment group, (n=6 or 7). Then, one animal from each treatment group was omitted from sperm count and testicular enzyme analyses with

the explanation that they were “growing” and in an attempt to exclude “exceptional incidence.” Moreover, no testicular histologic analysis was reported for one third of all males.

In light of these omissions and the curious rationale for dismissing some data that were collected, it is essential that the panel undertake a complete and comprehensive analysis of all raw data from the Mitsubishi marmoset study and note the limits imposed by lack of data availability before considering the results in the final evaluation of DEHP.

Page 113—Schilling et al.

Schilling et al. is an unpublished 2-generation reproductive toxicity study sponsored by BASF. DEHP exposure was continuous by diet. The draft NTP-CERHR report omits any discussion of histopathology provided in the Schilling study, yet microscopic findings are often the most sensitive indicators of toxic impacts of exposure to DEHP.

Testes were examined grossly and by light microscopy in the F0 parental generation and in the F1 generation after the offspring had reached adulthood.

In the F0 generation, two F0 parental females of the low dose (112 mg/kg/day) and two of the mid dose group (339 mg/kg/day) and three of the high dose group (1088 mg/kg/day) did not become pregnant. All control animals were pregnant. Schilling et al. conclude that the difference between the low- and mid-dose groups and controls was "spontaneous in nature" and not associated with treatment. They base that conclusion on the lack of corroborative findings in histopathology and the observation that the values are "within the range of historical controls." The authors did, however, attribute the fewer pregnancies in the high dose group to treatment, even though the numbers were within historical controls.

On histologic exam of the testes by light microscopy, Schilling et al. report that diffuse tubular atrophy was recorded in two high dose F0 males. Focal tubular atrophy occurred in one animal in the low dose group, three of the mid dose group, and six of the high dose group. One animal in the mid dose group also showed a few tubular giant cells. The authors attributed diffuse and moderate tubular atrophy to treatment with DEHP. However, without explanation, they concluded that focal tubular atrophy and the observed tubular giant cells were spontaneous in nature and unrelated to treatment.

Moreover, the authors noted that the mating of one pair of animals in the low dose group did not result in pregnancy, though the female was sperm positive. The male of this pair was found to have focal tubular atrophy. Yet the authors conclude that the fertility status of this male was unimpaired. The basis for that conclusion is completely missing, inasmuch as that male animal never sired offspring.

In the F1 generation, adult animals showed diffuse tubular atrophy in three high dose males. Focal tubular atrophy occurred in two animals of the control group, seven animals of the low dose group, four animals of the mid dose group, and thirteen animals of the

high dose group. Giant tubular cells were also seen in a few tubules of one animal in the mid dose group. Again, diffuse tubular atrophy and moderate or severe focal tubular atrophy were regarded to be treatment related. Minimal focal tubular atrophy and few tubules with giant cells were regarded to be of spontaneous origin.

Schilling et al. conclude from the results that the NOAEL for reproductive performance and fertility is 339 mg/kg/day and the NOAEL for systemic effects is 112 mg/kg/day (F0 and F1 parental males and F0 parental females) while below 112 mg/kg/day for F1 females (liver weight).

The Schilling study is limited in that the testes were examined only by light microscopy and not by electron microscopy which is a more sensitive technique. Moreover, it seems highly likely that the evidence of "focal" tubular atrophy in low dose animals of the F0 and F1 parental generations is simply an earlier stage of more advanced tubular atrophy, called "moderate" or "severe". The point at which subtle findings are considered treatment related as opposed to "spontaneous and unrelated to treatment" is unclear and subjective. Focal tubular atrophy and occasionally observed giant cells could easily be interpreted as treatment-related effects, in which case this study did not identify a NOAEL for DEHP using this protocol.

Summary:

In summary, Health Care Without Harm urges the NTP-CERHR expert panel to completely and comprehensively review the design, data, and interpretation of the Mitsubishi marmoset study and the Schilling rodent study if the panel intends to use those data in its evaluation of DEHP. The panel should consider the limits of each study (e.g. study power; study design provides no insight into impacts of fetal or infant exposures in marmosets), the authors' selection of data used/not used in the analysis, data interpretation, alternative explanations for individual variability, and the most sensitive endpoints, including histopathology.

The sections of the NTP-CERHR report "Strengths/Weaknesses" and "Utility (Adequacy) for CERHR Evaluation Process" should be revised to reflect those limits for these studies.

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